

PATENT SPECIFICATION

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(54) IMPROVEMENTS IN OR RELATING TO IMMOBILIZING ENZYMES AND MICROBIAL CELLS

5 (71) We, KANSAI PAINT COMPANY, LIMITED, a Japanese Body Corporate, of 365, Kanzaki, Amagasaki-shi, Hyogo-ken, Japan, and SABURO FUKUI, a citizen of Japan, of 7-203, Nagaokatenjin Haitsu, 4, Hachijogaoka 1-chome, Nagaokakyo-shi, Kyoto-fu, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

10 This invention relates to a method for immobilizing enzymes or microbial cells. More particularly, the invention relates to a method for preparing immobilized enzymes or microbial cells which are easily workable and are prepared by using hydrophilic photo-curable resin having two or more photopolymerizable ethylenically unsaturated groups per molecule.

15 In order to minimize the instability of enzyme activity and to facilitate continuous enzymatic processes, the technique to immobilize enzymes and to use them as solid catalysts has been recently employed in several industrial fields.

20 The immobilized enzymes may be prepared by the adsorption method, covalent bond method, cross-linking method and entrapping method. In the entrapping method, the enzymes are not bound to any matrix but are entrapped or microencapsulated in a fine lattice of gel. Therefore, the enzyme activity 25 of the product can be maintained effectively, and various kinds of enzymes and microbial cells can be treated by this method. In order to carry out this method, however, an immobilizing material which entraps enzymes or microbial cells without permitting release 30 and which has selective permeability to substrate, is required.

35 In the conventional entrapping method, aqueous suspensions of enzymes or microbial cells are mixed with low molecular weight hydrophilic monomers such as acrylamide, hydroxyethyl methacrylate, hydroxyethyl acrylate, hydroxypropyl methacrylate and

hydroxypropyl propyl acrylate, and the mixture is immobilized as it stands by polymerization. However, in this process, it is difficult to control properly the selective permeability of the obtained polymer matrix, so that the entrapped enzymes or microbial cells are liable to be released. In addition, the toxicity of the immobilized product prohibits its use in the food industry and pharmaceutical industry because unreacted low molecular monomers remain in the reaction product.

40 According to this invention there is provided a method of immobilizing enzymes or microbial cells, which method comprises substantially uniformly mixing an aqueous dispersion of enzymes or microbial cells with photo-curable resin having a number average molecular weight in the range of from 300 to 30,000, having two or more photopolymerizable ethylenically unsaturated groups per molecule and having nonionic hydrophilic groups and thereafter irradiating the mixture with actinic radiation.

45 This invention also relates to immobilized enzymes or microbial cells whenever immobilized by the method of the preceding paragraph.

50 A preferred method of this invention enables the provision of novel immobilized enzymes or microbial cells which are useful and economical from an industrial viewpoint since their activity is stable and the entrapped enzymes or microbial cells are well retained in the polymer matrix.

55 A further preferred method enables the provision of novel immobilized enzymes or microbial cells having various advantageous features, for example the immobilized enzymes or microbial cells can have good workability and no toxicity.

60 In a preferred method of this invention an aqueous suspension of enzymes or microbial cells and nonionic hydrophilic resin are mixed well and formed into a desired shape, then it is polymerized by irradiating actinic rays having a wave length of from 2,500 to 6,000 Å. The nonionic hydrophilic resin has a number average molecular weight of from

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300 to 30,000, preferably from 500 to 20,000, and two or more photopolymerizable ethylenically unsaturated groups per molecule.

5 The molecular weight of the photo-curable resin material is so chosen as to pass substrates but not to release entrapped enzymes or microbial cells. Preferably the resin is cured by actinic rays through a single operation to produce a mechanically stable immobilized product. This photo-curable resin preferably has hydrophilic groups to the extent that the resin mixes uniformly with the aqueous solution (buffer solution or the like) containing the suspension of hydrophilic enzymes or microbial cells. The activity of enzymes or microbial cells can be thus maintained in a most stable condition and the enzymes or microbial cells can be immobilized without the loss of their activity.

10 Furthermore, in contradistinction from methods employing gamma rays or electron beams, the degradation of activity of enzymes or microbial cells is not normally induced during the curing step since actinic rays are used.

15 In order to immobilize enzymes or microbial cells, two or more photopolymerizable ethylenically unsaturated groups are necessary for each molecule of the photo-curable resin.

20 When the number average molecular weight of the photo-curable resin is lower than 300, the cured product is liable to become brittle because the linearity of cross-linkage is low. On the other hand, when the number average molecular weight of the photo-curable resin is higher than 30,000, the viscosity of the mixture of the resin material and the suspension of enzymes or microbial cells becomes high and the workability of resin is impaired.

25 Therefore, the number average molecular weight of the photo-curable resin is within the range of from 300 to 30,000, preferably from 500 to 20,000.

30 The following advantages can be obtained over the conventional methods when the enzymes or microbial cells are immobilized according to a preferred method of the present invention. In this preferred method, a photo-curable resin having nonionic hydrophilic groups is first prepared from low molecular weights monomers and, subsequently the mixture of the previously prepared photo-curable resin and an aqueous dispersion of enzymes or microbial cells (hereinafter referred to as "enzyme-resin composition") is cured by irradiating with actinic rays. As a result, the dimensions of vacant spaces of the produced polymer lattices can be freely controlled and the liberation or releasing of enzymes or microbial cells can be prevented, which gives economical advantages. When the photo-curable resin is cured by gamma rays or electron beams, the activity of enzyme contained in the resin composition is reduced

35 because the energy of gamma rays or electron beams is very high. However, in this preferred method such a disadvantage is not normally induced since actinic radiation, such as ultraviolet radiation or visible radiation, is employed to cure the enzyme-resin composition. Therefore, in view of this, the activity of the enzymes can also be well maintained.

40 Furthermore, because in this preferred method the skeletal resin is first formed and thereafter the enzyme-resin composition is cured by a short time irradiation of actinic rays, lowering of the activity of the enzymes or microbial cells can be prevented as compared with the case when the polymers are prepared during irradiation of the enzymes or microbial cells solution from low molecular weight monomers, in which case relatively longer irradiation is necessary.

45 Still further, the steps of mixing the suspension of enzymes or microbial cells and photo-curable resin and irradiating actinic rays can be very simple. Furthermore, there is normally no necessity to handle low molecular weight monomers, which are generally toxic, during curing of the enzyme-resin composition and, therefore, the working environment in the curing step can be much improved.

50 When enzymes or microbial cells are immobilized using low molecular weight monomers according to the conventional method, the enzymes or microbial cells coexist with remaining monomers, so that the monomers must be removed by severe conditions of heating, acid or alkali treatment or organic solvent treatment. Thus, the activity of enzymes or microbial cells is seriously lowered by such treatment. In this preferred method, however, the remaining monomer can be easily eliminated in the step of producing the photo-curable resin, that is, in the step before entrapping enzymes or microbial cells. Therefore, the product prepared can be safely used in the food industry and pharmaceutical industry in which the toxicity of the remaining monomer is important.

55 Further, another advantage of this preferred method is that the work of formation or moulding of the immobilized product can be carried out without difficulty because the curing is performed by irradiation for a short period of time and the photo-curable resin is viscous to a certain extent. When the enzymes or microbial cells are formed into a membrane or a film, other resin can be mixed into the photo-curable resin in order to improve the mechanical strength of the film product, or strong bonds may be previously introduced into the photo-curable resin molecules. Therefore, the mechanical strength of the immobilized enzymes or microbial cells can be freely controlled. If required, the photo-curable resin solution containing the enzymes or microbial cells can be impregnated into or applied over synthetic or

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natural materials as substrates and then cured by the irradiation of actinic rays.

The below-defined photo-curable resins can be used in the present invention and some of which are exemplified in the following.

Photo-curable Resins:

Polyesters made from polyethylene glycol and acrylic or methacrylic acid:— diesters of unsaturated monocarboxylic acid made from acrylic or methacrylic acid and polyethylene glycol of from 400 to 10,000 in molecular weight containing less than 30% by weight of propylene oxide group; esterified products of unsaturated monocarboxylic acid made from 2 moles of acrylic or methacrylic acid and 1 mole of polyethylene glycol of from 600 to 10,000 in molecular weight; and esterified products of unsaturated carboxylic acid made from 3 moles of acrylic or methacrylic acid, n moles of tribasic acid such as trimellitic acid and

$$\frac{3}{2} - (n+1) \frac{2}{2}$$

moles of polyethylene glycol of from 600 to 10,000 in molecular weight.

Urethanated adducts of polyethylene glycol and 2-hydroxyethyl acrylate or methacrylate:— urethanated products made from n moles of diisocyanate such as tolylene diisocyanate, xylene diisocyanate, isophorone diisocyanate and hexamethylene diisocyanate, n-1 moles of polyethylene glycol having a molecular weight of from 800 to 10,000 and 2 moles of unsaturated monohydroxy compound such as 2-hydroxyethyl acrylate or methacrylate; urethanated products made from n moles of triisocyanate such as Desmodur L (trademark of Farbenfabriken Bayer A.G.), n-1 moles of polyethylene glycol having a molecular weight of from 800 to 10,000 and n+2 moles of 2-hydroxyethyl acrylate or methacrylate; and urethanated products made from 2 moles of diisocyanate, 1 mole of polyethylene glycol having a molecular weight of from 400 to 10,000 and 2 moles of unsaturated monohydroxy compound such as 2-hydroxyethyl acrylate or methacrylate.

Unsaturated cellulose:— adduct of water soluble celluloses such as cellulose acetate phthalate, hydroxypropylmethyl cellulose phthalate and hydroxyethyl cellulose with unsaturated glycidyl compounds such as glycidyl acrylate and glycidyl methacrylate or unsaturated acid anhydrides such as itaconic anhydride and maleic anhydride.

Unsaturated polyamide:— unsaturated polyamide made from adding the adduct of 1 mole of diisocyanates such as tolylene diisocyanate and xylene diisocyanate and 1

mole of unsaturated hydroxyl compound such as 2-hydroxyethyl acrylate, to water soluble polyamide such as gelatine.

The method of the present invention can be applied to various kinds of enzymes and microbial cells and they can be effectively immobilized without the loss of their activity.

In the following, the enzymes and microbial cells to which the present invention can be applied are exemplified. It should be noted, however, they are non-restrictive examples.

Enzymes:— urease, glucose oxidase, catalase, glucoamylase, glucose isomerase, invertase, glucose oxidase-catalase, lactase, D-amino acid oxidase, α -galactosidase, aminocyclase, aspartase and penicillin amidase.

Microbial cells:— those of *Lactobacillus bulgaricus*, *Aerobacter aerogenes*, *Bacillus subtilis*, *Azotobacter vinelandii* and *Proteus vulgaris*.

So as to promote the photopolymerization in the method of the present invention, commonly known photo-sensitizers can be added to the enzyme-resin composition.

Exemplified as such are α -carbonyl alcohols such as benzoin and acetoin; acyloin ethers such as benzoin methyl ether, benzoin ethyl ether, benzoin isopropyl ether, anisoin ethyl ether and pivaloin ethyl ether; α -substituted acyloins such as α -methylbenzoin and α -methoxybenzoin; polycyclic aromatic compounds such as naphthol and hydroxyanthracene; azoamide such as 2-cyano-2-butyl azoformamide; and metallic salts such as uranyl nitrate and ferric chloride. Further, mercaptans, disulphides, halogenides and dyestuffs can also be used.

These photo-sensitizers are used singly, or in mixture of two or more, in the range of from 0.2 to 7% by weight based on photo-curable resins.

In a preferred method of the present invention, the enzyme-resin composition is firstly formed into a desired shape and then the composition is irradiated with actinic rays. So long as the actinic rays reach the composition to be cured, the composition may be formed into any desired shape and thickness with or without a container. For instance, the composition can be applied on the surface of an article, laminated on another material, charged in a transparent container, impregnated into a porous material, or allowed to flow freely with irradiation. The articles to be applied with this composition may be of natural or synthetic products such as knitted or woven cloths or metallic products.

As the source of actinic ray irradiation, advantageously any instrument that gives out light rays in the range of from 2500 to 6000 Å in wave length can be used. Exemplified as such light sources are low pressure mercury lamps, high pressure mercury lamps,

fluorescent lamps, xenon lamps, carbon arc lamps and sun light. Advantageously, the irradiation time is generally from 1 minute to 10 minutes. It is advisable to irradiate with light rays in an atmosphere of inert gas so as to reduce the irradiation time.

In order that those skilled in the art may better understand the present invention and the manner in which it may be performed, the following specific examples are given. In the examples, unless otherwise indicated, parts and percentages are by weight.

Example 1

A uniform mixture was prepared from 90 parts of NK Ester 23G having a number average molecular weight of 1,135 (trademark of dimethacrylate of polyethylene glycol having a molecular weight of 1,000, made by Shin Nakamura Chemical Ind. Ltd. in Japan), 10 parts of 0.5% aqueous solution of glucose oxidase (containing catalase) dissolved in 0.1 M phosphate buffer solution at pH 5.6, and 1 part of benzoin ethyl ether. A platinum electrode was immersed into the thus prepared mixed solution and the electrode carrying the mixed solution thereon was irradiated at a temperature below 25°C for 5 minutes by a low pressure mercury lamp from the directions around the electrode, so as to form an electrode covered by immobilized enzyme. The above-mentioned electrode and another lead electrode as an opposite electrode were immersed in a 0.1% glucose solution and electrical measurement was carried out to observe the response of the enzyme electrodes glucose.

Example 2

Photo-curable resin (number average molecular weight 2,160) was prepared from 1 mole of xylene diisocyanate, 750 g of polyethylene glycol having a molecular weight of 1,500 and 1.1 mole of 2-hydroxyethyl methacrylate. Further, 85 parts of the thus prepared photo-curable resin, 15 parts of 0.1% buffer solution of urease, and 2 parts of benzoin methyl ether were uniformly mixed together. A glass plate of 3 mm in thickness was horizontally placed, on which a square frame (5 cm × 5 cm in inner dimensions) was formed with spacers of 1 mm in thickness and the above obtained mixture was poured into the inner frame on the glass plate. On the mixed solution, a polyester sheet of 0.5 mm in thickness was closely placed and then irradiated at a temperature below 35°C for 2 minutes by a 2 KW high pressure mercury lamp which was placed at a distance of 5 cm above, thereby forming a transparent immobilized enzyme product. This enzyme film was rinsed thrice with respective 200 ml of distilled water and immersed in 100 ml of 0.01 M urea solution prepared with 0.01 M phosphate buffer solution. The urea

solution was allowed to react for 30 minutes at 30°C. Then 5 ml of reaction solution was taken out and, after adding 5 ml of 0.1 N-HCl to the solution, it was subjected to back-titration with 0.1 N-NaOH. As the result of this test, it was estimated that the ratio of activity to that of the native enzyme was 68%.

Example 3

Photo-curable resin (number average molecular weight 2,160) was prepared from 1 mole of xylene diisocyanate, 750 g of polyethylene glycol having a molecular weight of 1500 and 1.1 mole of 2-hydroxyethyl methacrylate. Further, 65 parts of this photo-curable resin, 20 parts of NK Ester M-9G (trademark of methacrylate of methoxypolyethylene glycol having a molecular weight of 400, made by Shin Nakamura Chemical Ind. Ltd.), 1 part of benzoin ethyl ether and 15 parts of the solution prepared by dispersing 100 mg of glucose isomerase in 100 ml of buffer solution, were mixed together uniformly.

A glass-made test tube of 19 mm in outer diameter was concentrically placed in another glass-made test tube of 22 mm in inner diameter and the above mixed solution was poured in the space between both test tubes. Irradiation was then conducted at a temperature below 35°C for 3 minutes by using four 500 W high pressure mercury lamps from four directions perpendicular to the longitudinal walls of the test tubes, thereby obtaining a tubular transparent solid product. This tubular product was filled with 10 ml of 2% glucose-phosphate buffer solution containing magnesium ions and was kept at 50°C. The liquid which permeated through the tubular film was collected and the colour was developed by the cysteine-carbazole method so as to measure the formation of fructose by colorimetry at 560 m μ in wave length. As a result, it was estimated that 93% of glucose was converted into fructose.

Example 4

An enzyme solution was prepared by adding 50 mg of invertase and 50 mg of glucose isomerase to 10 ml of buffer solution at pH 7.0 and to this enzyme solution were uniformly added 90 g of NK Ester 23G (used in Example 1) and 1 g of benzoin ethyl ether. The above mixture was then poured into the inner frame used in Example 2 to 0.2 mm thick polyester film was closely placed on the mixture. From the above film, irradiation was carried out at a temperature below 35°C for 1 minute by using a low pressure mercury lamp to obtain an immobilized enzyme film. This enzyme film was cut into several pieces of 1 cm square and they were rinsed thrice with 1 litre of water. The cut pieces were then immersed into 100 ml of 2% sucrose solution (pH: 7.0) as substrate and it was

allowed to react for 60 minutes at 45°C. The produced fructose was analyzed using the cysteine-carbazole method and it was estimated that the ratio of activity to that of native enzyme was 45%.

Example 5

A uniform mixture was prepared from 90 parts of NK Ester 23 G (used in Example 1), 10 parts of *Proteus vulgaris* microbial cells) suspended in 0.1 M phosphate buffer solution at pH 7.0, and 1 part of benzoin methyl ether. This mixture was applied to a piece of gauze to form a 0.5 mm thick film and then, a 0.3 mm thick transparent polyester sheet was placed thereon. It was then irradiated at a temperature below 25°C for 3 minutes by a low pressure mercury lamp placed at 5 cm distance above and after the irradiation, the polyester sheet was peeled off to obtain an immobilized product of microbial cells containing the gauze as supporting structure.

Example 6

Photo-curable resin having a number average molecular weight of 4,675 was prepared from 1 mole of hydroxyethyl acrylate, 1 mole of isophorone diisocyanate and 2000 g of polyethylene glycol having a molecular weight of 4,000.

Further, 100 parts of thus prepared photo-curable resin, 300 parts of 2% buffer solution (pH 4.5) of glucoamylase, and 1 part of benzoin ethyl ether were uniformly mixed together.

Polyester film of 0.2 mm in thickness was horizontally placed, on which a square frame (5 cm x 5 cm in inner dimensions) was formed with spacers of 0.3 mm in thickness and the above obtained mixture was poured into the inner frame on the polyester film. On the mixed solution, a polyester sheet of 0.2 mm in thickness was closely placed and then irradiated at a temperature below 35°C for 3 minutes by 2 KW high pressure mercury lamp placed at a distance of 5 cm above, thereby forming a transparent immobilized enzyme product.

Ten pieces of this enzyme film (5 mm x 5 mm) was rinsed thrice with respective 10 ml of distilled water and immersed in 10 ml of mM maltase solution prepared with McIlvaine buffer solution (pH 4.5). The maltase was allowed to react for 30 minutes at 40°C. Then, 1 ml of reaction solution was taken out and the formed glucose was measured by glucostat which was manufactured by Worthington Biochemical Corp. As the result of this test, it was estimated that the ratio of activity to that of native enzyme was 65%.

WHAT WE CLAIM IS:—

1. A method of immobilizing enzymes or microbial cells, which method comprises substantially uniformly mixing an aqueous dispersion of enzymes or microbial cells with photo-curable resin having a number average molecular weight in the range of from 300 to 30,000, having two or more photopolymerizable ethylenically unsaturated groups per molecule and having nonionic hydrophilic groups and thereafter irradiating the mixture with actinic radiation. 65
2. A method according to Claim 1, wherein the enzymes are selected from urease, glucose oxidase, catalase, glucoamylase, glucose isomerase, invertase, glucose oxidase-catalase, lactase, D-amino acid oxidase, α -galactosidase, aminoacylase, aspartase and penicillin amidase. 70
3. A method according to Claim 1, wherein the microbial cells are selected from cells of *Lactobacillus bulgaricus*, *Aerobacter aerogenes*, *Bacillus subtilis*, *Azotobacter vinelandii* and *Proteus vulgaris*. 75
4. A method according to Claim 1, 2 or 3, wherein the number average molecular weight of the photo-curable resin is in the range of from 500 to 20,000. 80
5. A method according to any one of the preceding claims, wherein the photo-curable resin is at least one resin selected from polyesters made from polyethylene glycol and acrylic or methacrylic acid, urethanated adduct of the product made from polyisocyanate and polyethylene glycol with 2-hydroxyethyl acrylate or methacrylate, unsaturated cellulose and unsaturated polyamide. 85
6. A method according to any one of the preceding claims, wherein the wave length of the actinic radiation is in the range of from 2,500 to 6,000 Å. 90
7. A method according to any one of the preceding claims, wherein a reaction additive of polymerization initiator and/or photosensitizer is added to the mixture of photo-curable resin. 95
8. A method according to Claim 7, wherein the reaction additive is at least one additive selected from α -carbonyl alcohol, acyloin ether, α -substituted acyloin, polycyclic aromatic compound, azoamide, metallic salt, mercaptan, disulphide, halogenide and dye-stuff. 100
9. A method of immobilizing enzymes or microbial cells, substantially as described in foregoing Example 1. 110
10. A method of immobilizing enzymes or microbial cells, substantially as described in foregoing Example 2. 115
11. A method of immobilizing enzymes 120

or microbial cells, substantially as described in foregoing Example 3.

12. A method of immobilizing enzymes or microbial cells, substantially as described in foregoing Example 4.

13. A method of immobilizing enzymes or microbial cells, substantially as described in foregoing Example 5.

10 14. A method of immobilizing enzymes or microbial cells, substantially as described in foregoing Example 6.

15. Immobilizing enzymes or microbial cells whenever immobilised by the method of any one of the preceding claims.

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